# THE PREVALENCE OF VITAMIN B12 DEFICIENCY INTYPE 2 DIABETES MELLITUS PATIENTS ON METFORMIN

Dissertation submitted to

**THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY**

*In partial fulfilment of the regulations for*

*the award of the degree of*

**M.D. (BIOCHEMISTRY)**

**BRANCH – XIII**


**GOVT. KILPAUK MEDICAL COLLEGE AND HOSPITAL**

**THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY**

**CHENNAI.**

**APRIL-2016**

# CERTIFICATE

This is to certify that this dissertation entitled **"THE PREVALENCE OF VITAMIN B12 DEFICIENCY IN TYPE 2 DIABETES MELLITUS PATIENTS ON METFORMIN''** is the bonafide original work done by **Dr. D. REVATHI** Post graduate in Biochemistry, under the overall supervision and guidance in the Department of Biochemistry, Govt. Kilpauk Medical College, Chennai, in partial fulfillment of the regulations of **The Tamil Nadu Dr. M.G.R. Medical University** for the award of **M.D Degree in Biochemistry (Branch XIII).**

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# CERTIFICATE OF GUIDE

This is to certify that this dissertation entitled **"THE PREVALENCE OF VITAMIN B12 DEFICIENCY IN TYPE 2 DIABETES MELLITUS PATIENTS ON METFORMIN''** is the bonafide original work done by **Dr. D. REVATHI** done under my guidance submitted in partial fulfilment of the requirements for M.D. Branch – XVIII (Biochemistry) examination of **The Tamil Nadu Dr. M.G.R. Medical University,** to be held in April 2016.

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# DECLARATION

I solemnly declare that this dissertation entitled "**THE PREVALENCE OF VITAMIN B12 DEFICIENCY IN TYPE II DIABETES MELLITUS PATIENTS ON METFORMIN"** is the bonafide work done by me in the Department of Biochemistry, Govt. Kilpauk Medical College, Chennai, under the guidance and supervision of **Prof. Dr.V. MEERA, M.D.,** Associate professor of Biochemistry, Govt. Kilpauk Medical College, Chennai–600 010.

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# CONTENTS

# ABBREVIATIONS

| | | |
|---|---|---|
| ADA | - | American Diabetes Association |
| WHO | - | World Health Organization |
| GLUT | – | glucose cotransporter |
| T2DM | - | type 2 diabetus mellitus |
| IDF | - | International Diabetes Federation |
| CBL | - | cobalamine |
| DCCT | - | Diabetic Control and Complications Trial |
| GLP | - | Glucose - dependent Insulinotropic Polypeptide |
| GLP-1 | - | Glucagon - Like Peptide-1 |
| NGT | - | Normal Glucose Tolerance |
| MBL | - | methyl cobalamine |
| ADO CBL | - | adenosin cobalamine |
| MMC | - | Methyl melanoyl coA |
| HCY | - | Homocysteine |
| CIE | - | Ca2+dependant Insulin Exocytosis |
| cAMP | - | cyclic Adenosine Mono Phosphate |
| PKA | - | Protein Kinase A |
| PG | - | Pro-Glucagon fragment |
| IP-1 | - | Intervening Peptide-1 |
| IP-2 | - | Intervening Peptide-2 |
| gcg | - | glucagon gene |
| LRP | - | Lipoprotein Receptor-related Protein |
| Ryk | - | Receptor tyrosine kinase |
| TLE | - | Transducin-like Enhancer |

# THE PREVALENCE OF VITAMIN B12 DEFICIENCY INTYPE 2 DIABETES MELLITUS PATIENTS ON METFORMIN


## ABSTRACT

**Introduction:**

Diabetes Mellitus is the most common endocrine disorder and Metformin is the most commonly prescribed oral hypoglycaemic agent. Metformin is well known to cause vitamin B12 deficiency due to effect on calcium-dependent membrane action in the terminal ileum leading to malabsorption of vitamin B12. The purpose of this study is to determine prevalence and associations of Vitamin B12 deficiency in patients of type 2 diabetes mellitus treated with Metformin.

**Methods:** This cross sectional study was carried out in department of diabetology, Combined department of Biochemistry from 1st February 2015 to 30 August 2015.We enrolled 45 outdoor patients of type 2 diabetes mellitus currently on Metformin for at least 12 months, by consecutive sampling, and 45 age and sex matched patients on other drug were taken as control. Patients with vitamin B12 levels of less than150 pg/ml were said to be B12 deficient. The results were analysed on SPSS version 16. **Results:** Serum B12 levels were low in 17 patients (22%) on Metformin as compared to only 9 patients (8.6%) among controls,(p value 0.002). Mean B12 levels were significantly low in

Metformin group  311 pg/ml (±194.4), p value 0.03. Dose of Metformin had inverse correlation with B12 levels and the difference was statistically significant with p value <0.001.

**Conclusion:** Our study demonstrated significantly high prevalence of vitamin B12 deficiency in patients treated with Metformin.

Significant effect on dose and duration of Metformin use on B12 levels. Physicians must recognize this important fact and screen diabetics' on Metformin therapy for underlying B12 deficiency.

# INTRODUCTION

Diabetes mellitus is rapidly emerging as a current pandemic in this century.[1] According to International Diabetic Federation 2014; nearly 183 million people are still unaware that they are living with diabetes. Therefore the identification of individuals at high risk of getting diabetes is of great importance for investigators and health care providers.[2] The target is to reduce the prevalence of the disease and its economic burden and enhance quality of life for all persons who have and are at threat of Diabetes Mellitus.

It has equal priority in both developed and developing countries. It is attracting the world since the global crisis due to diabetes cripples not only the health but also the economy of every country. The glad news is that once the risk factors are accessed the development of Type 2 Diabetes can either be deferred or even prevented by healthy customs.

The Greek Apollonius of Memphi first used the term "diabetes" or "to pass through" in 230 Bc[1]. [(2)] The Indian physicians, Sushruta and Charaka were the first to identify Type 1 and Type 2 Diabetes as two separate conditions. In the late 17$^{th}$ century Britain John Rolle added the[2] term "mellitus" or "from honey" to separate the condition Diabetes insipidus.

Diabetes - a multisystem disease due to defect in metabolism of glucose which causes multiple irregularities in the metabolism. Metabolism of glucose is well organized by multiple hormones and neurotransmitters in response to nutritional[3], emotional and environmental changes. Unger, first labelled diabetes, as a "bi-hormonal" disease.[3]

Conventionally the pathophysiology of T2DM was engrossed on beta cell dysfunction and insulin resistance in liver and skeletal muscle. Numerous researches in the past two decades exposed a basic understanding about mechanism and dysfunctions in gastrointestinal tract, pancreatic alpha cells, adipose tissue, brain and kidney that produced a more tough picture of Type 2DM[4]

Many studies in pathology of the disease had introduced the novel drugs alike pancreatic-protein-coupled fatty-acid-receptor agonists inhibitors of the insulin-releasing glucokinase activators, 11ß-hydroxysteroid dehydrogenase, sodium-glucose cotransporter 1, glucagon like peptide-1 analogues, glucagon-receptor antagonists and quick-release bromocriptine, dipeptidyl peptidase-IV inhibitors, and metabolic inhibitors of glucose output from liver [5]

Diabetes is a multifaceted process and includes managing disease complications, drug related adverse effects in addition to glycemic

control. There are supporting evidences that various parallel interventions improved clinical outcome in these patients. Though so many novel drugs are available, the incidence of Type 2 DM is still in the advanced end.[6]

The American Diabetes Association advises biguanides like metformin must be the primary therapy forT2DM .When used alone it rarely causes hypoglycaemia. It increases sensitivity to insulin and increases weight loss and alters lipid profile[7]reasonably. Metformin acts through [53] enhancement of activated protein kinase of adenosine monophosphate (AMPK) system to decrease sugar levels in the blood. The main activity of the drug is on gluconeogenesis[9].in the liver

Adverse events of the therapy are intestinal disturbances and, seldom disturbances in the muscle gluconeogenesis called lactic acidosis in addition to vitamin B12 deficiency which is commonly overlooked and the monitoring is for clinical side-effect[1011], Pflipsen *et al. i* indicated that 22% of cases had a B12insufficiency in 2009, and person who are prescribed the above drug had reduced vitamin B12 levels.

While Lactic acidosis manifests only in the setting of heart failure, renal failure and alcoholism, it is uncertain whether vitamin B12 malabsorption is due to DM itself or to biguanides [8]

Ting *et al.* publicized that if the drug advised for chronic period it causes decreased B12 levels. It is dependent on the amount of the drug.

The mode action of the drug is unknown. It is mainly by changing the movement of the intestine or intracellular handling of calcium, thereby reducing absorption of vit.B12. It is accepted that calcium restores the deficiency[10].

It is significant to identify the impact of B12 insufficiency. B12 is necessary for, cellular repair DNA synthesis and for the regular synthesis of RBC [912]

[13] B12 is necessary for the metabolism of dopamine, monoamines and serotonin. Because of vitamin B12 deficiency, all of the above neuro transmitters' synthesis will be deficient which collectively end in neurocognitive or psychiatric manifestations, Axonal degeneration demyelination and neuronal death. Vitamin B12 deficiency induced neuronal damage manifests as autonomic neuropathy and peripheral neuropathy sub- acute combined degenerate ion of the spinal cord,[13 14]

Chronic metformin use results in reduced level of VitB12 which can exacerbate or cause peripheral neuropathy due to DM. Because of the action of the glycation end products on vascular endothelium diabetics are more vulnerable for diabetic neuropathy. Unluckily the manifestations of diabetic neuropathy overlap with the paraesthesia impaired vibration sense, and impaired proprioception related with Vitamin B12deficiency.[1314]

As a consequence, B12 deficiency–induced neuropathy may be confused with diabetic peripheral neuropathy. Recognizing the exact cause of neuropathy is crucial, because simple vitaminB12 supplementation may revert neurologic symptoms improperly attributed to hyperglycaemia

# AIMS AND OBJECTIVES

Metformin and life style modifications are the first line therapy in type 2 patients as per the ADA. It is well tolerated by most of the type 2DM persons. But the main adverse effect is vitamin B12deficiency which is almost forgotten and the viaminB12screening is rarely advised

Primary aims of the research are to identify “the prevalence of vitaminb12 deficiency in T2DM persons on metformin”, and compare them with those patients receiving other hypoglycaemic agents. The secondary objective is to advise base line screening of vitamin B12 before starting the drug.

# REVIEW OF LITERATURE

In the 21st century next to China, India leads the world with the most number of Diabetics aptly termed as Diabetic capital of the world. In 2000 31.7 million people suffered from Diabetes but in 2015 it is >62 million. China has an incidence of 20.8 million, in USA it is17.7 million, where as in INDIA it is71[18] million. The global incidence of DM is 366 million and the chief contributor is India.

By 2030 the incidence of type2 DM in India will be 79 million and for china it is42.3million and for US it will be30.3million. Rendering to new valuations, approximately 285 million individuals worldwide (6.6%) in the 20–79 year age crowd will show diabetes in 2010 and by 2030, 438 million people (7.8%) of the mature people will have the disease.

In India, the persons in rural and urban areas were equally affected by diabetes; Urban India has a higher incidence of 5.6% than Rural India with 2.7. The high prevalence observed in urban area shows that the rapid urbanization plays a major role. The most worrying trend in incidence of diabetes is its shift towards younger age where Diabetes commences ten years earlier than in western countries[20]. In Chennai, diabetic prevalence was 13.5% in 2000 that went up to 14.3% in 2004 and reached 18.6% in 2006[20].

The increased prevalence of type 2 DM in urban regions is due to the fact that people are having easy access to diagnosing diabetes. In rural regions food insecurity, dominance of communicable diseases, illiteracy, lack of counselling, poor sanitation, inadequate infra-structure in screening, and long travel to reach health care facilities [20] are the reasons for the decreased prevalence.

Quite a lot of studies on migrating Indians across the world have revealed that Indians having a greater risk of developing T2 DM and metabolic irregularities matched with other racial groups. Though the primary cause for these problems is still vague, some unique biological variables of this race called as the "Indian phenotype"- was thought to be one of the main causes accounting to the increased tendency towards type 2 diabetes, in spite of low incidence of obesity.

Increased abdominal obesity in Indians is due to more waists to hip ratios and waist circumference. Again, in Indians for any given BMI there is greater total abdominal, visceral body fat. So increase in the incidence of insulin resistance occurs. They also have reduced levels of the protective adiponectin and adipokine and have raised metabolites of adipose tissue. Studies on newborn indicate that Indian babies are small at birth but fatter when compared to babies of other races and are mentioned as "the lean fat Indian babies".

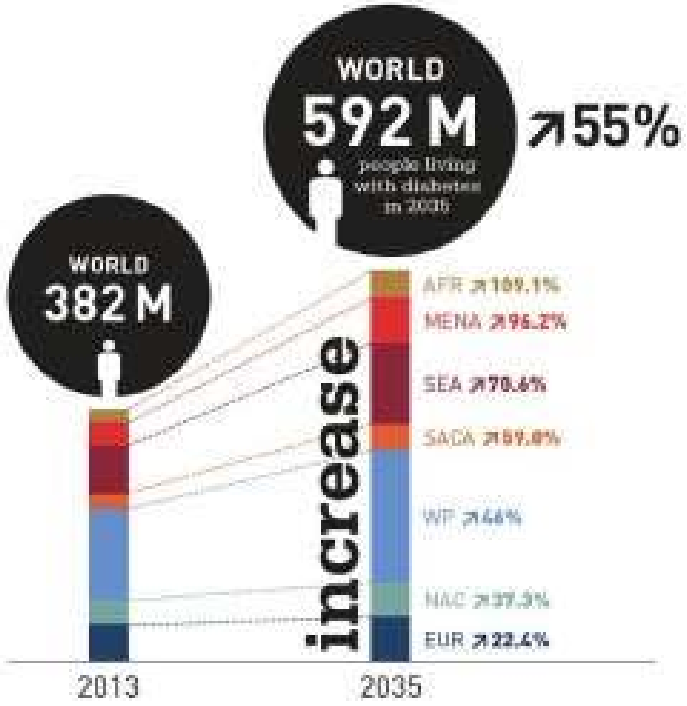
A recent study concluded this finding and has found that the “lean fat phenotype” in newborns was present throughout the life of the Indian child and this would lead to the diabetogenic adult. These results propose that Indians are vulnerable and predisposed to become diabetics with associated metabolic complications.

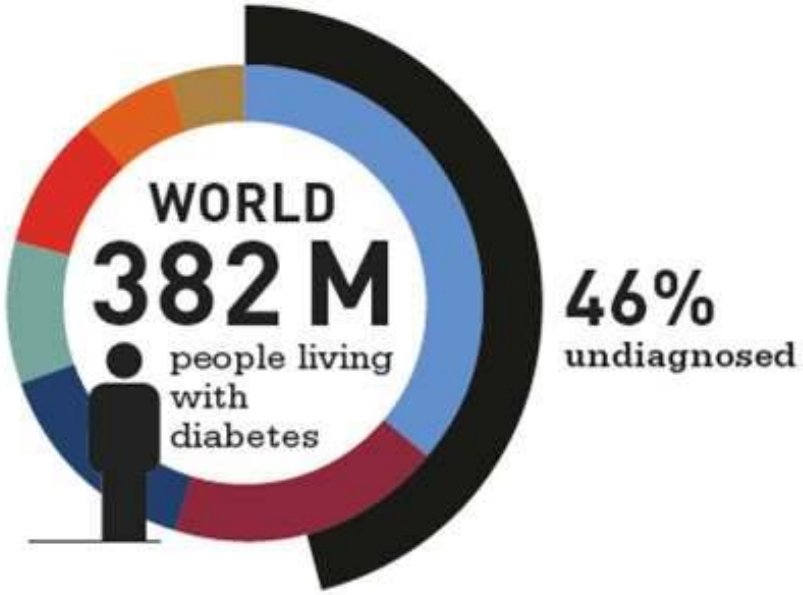
Genetic variables that control fat distribution and metabolism of glucose need to be explained for the better understanding of the pathogens is of diabetes. These studies have concluded that certain genes are responsible for the increased prevalence of diabetes in Indians. Few genes that are protective for the Europeans do not have the same action in the Indians.

**The epidemiological transition of Type II DM**

The intense rise in the incidence of type 2diabetes and the connected disease associated with it for example, obesity, hypertension and the syndrome x might be linked to the fast life style habits during the last 50 yrs. Though this” epidemiological transition”[22], which comprises better-quality nutrition, improved hygiene, control of several infectious diseases and better-quality access to quality health care have ended in prolonged expectancy, which causes the current diseases like obesity, diabetes and cardiac diseases. Western life style introduction into these

societies led the shocking trends in obesity and the linked metabolic complications.



According to NEEL THRIFITY hypothesis the specific genes designated in the former millennia (Indians) permitted survival during the starvation by powerfully glowering all energy obtainable during times of festival. This genetic factor leads to type 2DM and obesity when bare to continuous high energy diet. [20]

The modern trend, adolescent's children and young adults are the victims for Type 2 diabetes and pre diabetes in countries like Japan, USA, India, Australia and UK. According to Bloomgarden et al., the earlier the age the greater is the burden, as the major complications fall on the future generations.

Diabetes is meant by a group of metabolic defects of variable etiology which is branded by overt hyperglycaemia and other abnormalities of glucose metabolism due to deficiency of insulin, insulin secretion or both. Major forms of diabetes are; type 1 and type 2.

Deficiency of or severe reduction in insulin secretion due to immunological or viral destructions of ß cells is the reasons for type 1 diabetes, which credits for 5-10% of diabetic populations. The more predominant form, DM 2, accounts for more than90% of cases (Olefsky, 2001).[20]

Diabetes type 2 typically[20] starts as resistance, to insulin. In this disorder the insulin is not properly used by the cells. As the insulin need is increased, the pancreas loses its ability progressively. Deficit of insulin action and/or secretion brings hepatic glucose output by inhibiting glycogen synthesis and stimulating glycogenolysis and gluconeogenesis. This particularly results in fasting hyperglycaemia (Defronzo an Simonson, 1992 and Michael et al, 2000)[20]

## CLASSIFICATION

WHO (1999) published a revised classification which is mostly followed. This covers both clinical and etiological types of diabetes mellitus.

**Diabetes Mellitus - Etiologically classified into four categories**

**I. Type 1 Diabetes** (typically show absolute insulin deficiency since the β cells are destroyed).

A. Idiopathic

B. Immune mediated.

**II. Type 2 Diabetes**

This is due to insulin resistance along with virtual insulin β cells deficiency or by defects in the secretion mechanism.

**III. Other Types**

A. Genetic defects in insulin action

B. Diseases of the exocrine pancreas

C Genetic and Epigenetic defects of β- cells

D. Drug or chemical induced

E. Endocrinopathies

F. Uncommon forms of immune-mediated diabetes

G. Infections

H. Other genetic syndromes sometimes associated with diabetes

**IV. Gestational diabetes mellitus.**

Clinical staging of T2DM

1. Normoglycemia
2. Impaired Glucose Regulation- and Impaired Glucose Tolerance (IGT)
3. Diabetes mellitus
4. Impaired fasting Glycaemia(IFG)

The majority of cases drop into first two classes of diabetes

**Pathophysiology behind Diabetes:**

Diabetes is a condition where, chronic elevation of blood sugars is seen. The chief defect is in the metabolic pathway of carbohydrate. Carbohydrates are not only the main energy yielders they also needed for exact cellular purposes and protein alterations by glycosylation. Therefore the body has to sustain the blood glucose levels within narrow limits which are maintained by many hormones of which, the important action is through Insulin.

Insulin is the chief hormone of blood glucose regulation.. Insulin rises glucose level by promoting gluconeogenesis in peripheral tissues, also promotes lipogenesis, cell growth and differentiation. It also enhances protein synthesis glycogen synthesis, glycogenolysis, lipolysis and protein interruption in liver and muscle. So continuous deregulation in the action or secretion of these processes and causes the changes in the fasting lipid and fasting glucose levels.

**RECPTORSFOR INSULIN SIGNALLING**

Insulin action starts by attachment of the hormone to exact insulin surface receptors. Insulin receptor Coding Gene is INSR.[19] This receptor possess bi functional glycoprotein subunit domains: an extracellular alpha-subunit covering at most hormone binding site and beta subunit

with intrinsic tyrosine kinase activity.[20] Insulin provokes a wide-ranging biological replies by attaching to its exact receptor. Attachment of insulin to the alpha subunit creates a conformational change which ends in the auto phosphorylation of a sum of tyrosine residues existing in the beta subunit [21.30] Insulin receptors trans phosphorylate several subsequent substrates including –and Gab 4, IRS1 1, Cbl, and P60dok APS, [31]. After phosphorylate ting to tyrosine kinase, the above mentioned molecules connect with over their SH2 domains, produces a mixed series of signalling ways of the concerned hormones.

i. Ras and MAP kinase cascade

ii. Activation of PI(3)K/Akt pathways

iii. Cbl/CAP These pathways performances in a harmonized way to control glucose, lipid and protein metabolism

**Akt pathways & PI3 kinase**

PI3 kinase plays a main role in the regulation and in the expression of insulin hormone. The heterodimer PI3 includes of the p85 regulatory subunit and p110 catalytic subunit. Activated PI3K go and activates, PIP2, PI3P and PIP3respctively. The phospholipids proteins are reported as the second line conveyers of message. This protein operates with a help of threonine kinases and serine[3, 4, 5] kinases. These proteins transport

Akt to the membranes from cytsol via attaching with "pleckstrin homology domain kinases.[3.] Transport of the lipids and the attachment of receptors create differences in charge. With the help of PDK1, Akt activities are stimulated by means of phosphorylation.

If Akt phosphorylation is initiated it controls the many regulator proteins which are followed in the succeeding path ways of cellular function of carbohydrate metabolism. Amongst others, Akt phosphorylates and panels components of the, protein kinase C (PKC) isoforms GSK3and glucose transporter 4 (GLUT4) complex all of are `vital for assisting the biological activities of the hormone of insulin[34,35]

**Insulin- MAP kinase pathway**

Insulin excites the of mitogen kinases protein present in this path which are already stimulated. Because the mitogen kinas are activated it results in the phosphorylation of IRS and tyrosine kinases proteins .Next it stimulates the connector Grb2 to, recruiting the Son-of-seven less (SOS) interchanger enzyme to the membrane. which in turn go and activates the Ras.[37] After the activation of the tyrosine phosphatases then SHP2 lastly the Ras are stimulated. Since the activated Ras are connected with receptors like IRS1/2or Gab-1. As soon as stimulated, Ras works as a biological switch, exciting a chute of serine kinase like chain of action follows MEK Raf, and ERK. Activated ERK will trans -lodged to the

nucleus, where it causes of transcription and phosphorylation of essentials such as TCF, p62 prompting a cellular proliferation or differentiation[31,]

**Insulin resistance & Type 2 diabetes mellitus**

It is demarcated as a defective response in target tissues such as the skeletal muscle, liver, and adipocytes, to act of normal level of insulin. Current situations insulin resistance plays major role in the developmental stages of T2DM. Mainly to overcome this situation endocrine system produces the concerned hormone which allows the person for better withstanding of regular glucose Level.[40] During the period of time, progressive loss of cells and incapability of pancreas causes a condition absolute insulin insufficiency and later the stage of T2DM.. This resistance to insulin is the chief issue leading to the pathophysiology - T2 DM[41].

**MECHANISM BEHINDTHE DISEASE**

Several mechanisms has been showed as reason for insulin resistance,

1) **Insulin sustains blood sugar within a very critical range.**

Raised glucose load, glycaemic fluctuations and the pancreas is enforced to over-secrete insulin. Continuing hyperglycaemia roots to failure of the beta cells of endocrine pancreas to secrete insulin and later may cause Type 2 Diabetes Mellitus. Eventual analysis shows that

normal glucose tolerant person with a elevation of pancreatic glucose burden have an better risk of emerging Type 2 diabetes compared to person with a low pancreatic glucose level. Mechanisms consist of reduction in expression of related genes (GLUT2insulin receptor, rectifier potassium channel, glucokinase,) beta cell differentiation in addition to greater risk of apoptosis.[41]

**2. Lipid Accumulation liver & adipose tissues**

Increased lipid levels in skeletal muscle and, augmented liver triglycerides are the root cause of insulin resistance. Increased insulin level in the blood is complicating the lipid synthesis in liver and muscle is promoted through SREBP1c - regulatory element-binding protein 1c representation at the genetic level.[43] The chief regulator in de novo lipid synthesis at genome level is SREBP1c - l. Liver, adipose tissue and skeletal muscle, since the lipoprotein lipase is over stimulated, lipids getting accumulated and leads to resistance. Plentiful studies point out that chronic exposure of the pancreas to elevated FFA levels has harmful effect on the beta cells[44].

**Insulin Resistance by FFA**

In skeletal muscle, accumulation of lipid will ends in raised ratio of NAD /$NADH^+$ acetyl CoA/CoA –in the mitochondrial level. This ends in the PDH (pyruvate dehydrogenase) inhibition. These causes , elevation in

the citrate level in the carbohydrate metabolism. This in turn causes phosphorylation of phosphofructo kinase so the glucose-6-phosphate levels are raised at the cellular level. This will deactivate the enzyme hexokinase II ending in raised glucose levels in the cells and reduced glucose utilisation in the muscle.

[46]Another explanation is given for the lipid accumulation and subsequent development of resistance in muscle is accumulation of metabolites of lipids in the cells such as, fatty acyl CoA, ceramides and diacylglycerol. This lipid and lipid metabolites accumulation within skeletal muscle and liver activate serine kinases cascade through PKC-ε, reduced insulin signalling at receptor level. Decreased, tyrosine and IRS-2– activity ends in phosphorylation of PI 3- kinase,

[47] Defective IRS-2 either in molecular or in the site of action level reduces the activity of glycogen synthase, this in turn causes decreased glucose absorption and decreased liver gluconeogenesis. The above mentioned reaction is insulin dependent defective PI-3 mediates defective activity of GLUT-4 in the membrane.

**Low AKT2 activity**

[49]Lowered activity of AKT2 results in dec reased phosphorylation of fork head box protein O (FOXO), letting it to enter the nucleus and trigger the transcription of the rate-controlling enzymes of

gluconeogenesis (carboxykinase, glucose-6-phosphate phospho-enolpyruvate phos-phatase). Amplified gluconeogenesis further worsens hepatic insulin resistance and results in fasting hyperglycaemia [50]

## D. PHARMACOLOGICAL AND OVERALL APPROACHES TO TREATMENT OF DM (ADA-2013)

### 1. Remedy for type 1 diabetes

**Guide lines**

1. Maximum individuals of type I diabetes are managed by multi dose insulin injections -three to four injections of basal and prandial insulin or constant insulin infusion of subcutaneous type -CSII

2. Al most all type 1 diabetics are explained about the increase or decrease of insulin dose according to fasting and post prandial blood glucose level and also the expected outcome.

3. Many of the diabetic of type 1 diabetics are advised to use analogues of insulin to decrease hypoglycaemia complications.[50]

4. Immunological screening is advised to do in the expected cases.[51]

5. Short- and intermediate-acting insulin is recommended in the initial stages irrespective of micro vascular complications. Exhaustive insulin treatment was associated with unexpected

hypoglycaemia .Out of hundred, sixty two cases will have severe hypoglycaemia. Meanwhile many long acting and rapid acting preparations are available. These are having less adverse events. Treatment is individual based and also dependent on the micro vascular status of the individual. Strict glycaemic status is followed.

6. Insulin analogues are used in cases of or recurrent hypoglycaemia. Many guide lines are available regarding tight glycaemic practise. Since many of the type 1 DM are due to auto antibodies. So screening for thyroid antibodies, pernicious anaemia, coeliac antibodies are advised.[51]

7. **Therapy for hyperglycaemia in type 2 diabetes**

Metformin is first drug of choice forT2DM. In the absence of contraindications, if well tolerated it is the primary drug of choice.

1. Newly diagnosed T2 DM having definite signs and high levels of A1C, insulin is recommended either as single drug or with other OHAS.

2. If severe hyperglycaemia persists after six months of single drug therapy, another drug like GLP-1 included.

Reduction of basal liver gluconeogenesis within the physiological limit is the expected outcome of the diguanides. This activity is combined

with the inhibitory role in the electron transport chain is, later proved in the literatures. The lactic acidosis which is the most serious adverse event of the drug is mainly due to the mitochondrial inhibition rather than therapeutic effects.[55]

Metformin has minimal influence on ATP levels of the cells, even when using in more concentration. The sugar reducing activity is chiefly due to the inhibitory effect on the complex I of the electron transport chain. This is individualised for every diguanides which are already exists.

The effect on mitochondrial ETC, the decreased oxygen consumption and Combining the TCA cycle for ATP which fulfils the energy needed for the every activity of each cells in the body.

This is proved in a study which was conducted in the year 2000. Studies on, freeze-clamped livers hepatocytes showed that metformin reduces the basal liver glucose output by inhibiting complex I in the mitochondrial electron chain.

The biguanides blocks the oxidation of glutamate and malate in the citric acid cycle more effectively. This proved that the target place for metformin is the complex I of ETC. Succinate a substrate which acts as a

complex II can bypass complex I action of metformin. Similar researches completed before by other guanide-containing drugs also[66].

This study gives evidence of association between reduction in glucose output and inhibition of complex I in electron transport. But it is not genetically proved. So to prove the site action of metformin more researches are needed

Complex I inhibition also gives a correct explanation of lactic acidosis in metformin treatment. Lactate levels increased by glycolysis should be expected to complex I inhibition comparing with buformin and phenformin, lactic acidosis is less common with metformin. This metformin-dependent mitochondrial inhibition is assumed to be self-limiting as the mitochondria is active.[68]

**The Mechanism of Activity**

The mechanism defined for the drug action is reduction in the basal hepatic output of glucose. The T2DM patients have much more gluconeogenesis than normal. Metformin reduces this to one-third. It activates the first component of ETC in the mitochondria since the protein kinases stimulate the cyclic AMP. This is done with help of glucagon. Because of the activated protein kinase the mitochondrial glycerophosphate dehydrogenase activity is reduced .[69]

AMPK, is having important activity in the various metabolisms like protein and fats and its chief activity is concerning with the insulin hormone signalling. It is needed for metformin's inhibitory action on complex I. Stimulation of AMPK is essential for the improved representation of the small heterodimer, which go and suppress the representation of the important enzymes like glucose 6 phosphatse, corboxy kinase, and phosphoenal pyruvate kinase at the genetic level. The biguanides action on AMPK remains unknown.

[70]Raised cellular level of AMP also been proposed for the raised cAMP by glucagon and, also the activated kinase of protein system activity is antagonized by metformin. Thus reduces the fasting hyperglycaemia. Metformin also brings a strong alteration in the faecal micro flora in diabetic mice, and it may be a consequence of GLP 1like activity.

[71]Metformin not only controls hepatic gluconeogenesis, also increases sensitivity to insulin. It stimulates the activity of GLUT -4 in the peripheral tissues by phosphorylation. It also causes reduced fatty acid oxidation which is mediated by insulin and also reduces the uptake of glucose from the intestines. By enhancing the activity of insulin to its receptor it stimulates more utilisation of glucose in the peripheral tissues. This is also proved in type 1DM also

Activated kinases are having primary role in the function of the biguanides. Activated kinases, promotes dislocation of GLUT receptors in the intestinal membrane which reduces the glucose uptake in the absorption levels itself. Some activities of the drug are without the involvement of phosphate kinases. This is proved in heart muscle.[71]

**Pharmacokinetics**

In fasting Conditions the oral bioavailability is 60%.The absorption is slow. Three hours after the intake, maximum concentration of plasma is achieved in the regular formulas. Seven to eight hours in the sustained release.[72]

In the body PH it is having the pKa of 2.8 -11.5. The p Ka makes the drug a harder base. It is less soluble in lipids. It is diffuses passively in the membranes of plasma. The double methyl substituent of metformin offers minor lipid solubility than phenyl ethyl side chain in its other drug phenformin. The lipophilic character of metformin is used for producing prodrugs with better oral bioavailability than metformin[59].

Tubular excretion is the mode of clearance from the body. In the urine it is eliminated without any changes. After 24 hours of intake it is not detected in the plasma. Normally it is eliminated from the plasma within 6.2 hours.

## Contraindications

The drug is contraindicated in chronic kidney disease persons. (If CR is more than 1.7) It is contraindicated in chronic liver disorders, progressive parenchymal diseases of the lung and also in congestive cardiac failure. Since cardiac failure persons may develop the fatal condition called lactic acidosis.

Metformin is temporarily withdrawn before any radiographic study including iodinated contrast agents, such as a contrast-enhanced CT scan or angiogram, as the contrast dye may induces short term impairment in kidney function, ultimately leading to lactic acidosis by initiating retention of metformin in the body. Metformin is restarted two days after the investigations.[61]

## Adverse effects

1. The most common effect is gastrointestinal upset like diarrhoea, cramps, nausea, vomiting, and increased flatulence.
2. The most severe and rare side effect is lactic acidosis. The vast majority of these cases are related to other complication such as liver or kidney dysfunction, rather than to the drug itself.
3. Metformin reduces the levels of thyroid-stimulating hormone in hypothyroidism persons.

**Gastrointestinal**

1. Gastrointestinal upset can cause severe discomfort after the first dose. If the drug is started with a minimal tolerable dose this adverse event is overcome. The dose should be adjusted progressively

    - The most severe adverse event of duiguanides is lactic acidosis -MALA- metformin-associated lactic acidosis.

    - Prevalence MALA is 9/ 100,000 human-years.

    - The lactic acidosis – that is lactate taken to the hepatic tissue is reduced while using metformin, since the source for gluconeogenesis is lactate, a route in which the drug survives. But this is seen more in phenformin.

**Interaction**s

The $H_2$-receptor blocker cimetidine raises the plasma levels of metformin. The two drugs were eliminated from the kidney by the mechanism called - (tubular secretion). Predominantly the clearance of cations.Both the drug competes through same transportation [62]

One case control study revealed cephalexin an antimicrobial drug also increases the duiguanides levels by the same way.

## OTHER THERAPEUTIC USES

1. Pre diabetes
2. Polycystic ovary syndrome
3. Gestational diabetes
4. Anti cancer activity

### Pregnancy

In GDM (gestational diabetes) and also in pregnant diabetics the safest drug is metformin. When the drug is prescribed, the mothers had less weight gain comparing with mothers on insulin. Neonates of mother who received metformin had minimal visceral adiposity .Since the future obesity related disorders are reduced in these children.

### Researches

Duiguanides specifically used in modules of ovarian, pancreatic, lung, a prostate breast, colon cancer cells. To test its efficacy in antitumor tissues studies are going on. The first clinical trials confirmed an advantageous effect in breast and colon cancer .Inhibiting action of the drug over the first component of respiratory chain increases the potency of radiotherapy by reducing free radicle formation.

Although it is used in various diseases the use of the drug in type 2 DM is unbeatable so far. Sustained use for long duration causes elevated homocysteine level in blood and also raised rate of vitamin $B_{12}$ deficiency, so the investigators are advised to screen for the vitaminB12 deficiency annually.

**THE EVOVLUTION OF B12**

[64] Pernicious anemia when it was discovered, a fatal condition, $B_{12}$ was the cure which was revealed accidently. The researches in anemia were conducted by the great man called GEORGE WHIPPLE, he used to give different types of foods to dogs after bleeding them to see which type of food allow them fast relief from the anemia produced

He noticed that liver in large amounts cured the anemia caused by bleeding. Finally he came to know the truth liver extract corrected the B12 deficiency anemia.

Later on after several studies George Richards Minot and William Murphy found a separate compound of hepatic origin corrected the vitamin deficiency in dogs, and they thought it may be iron. Later the team came to know the truth the compound corrected the bleeding disorder in dogs and human are entirely different one. The liver extract which cured pernicious anemia was discovered like this. Minot and

Murphy explained this in the year 1926. This is the evaluation of vitaminB12[65].

Edwin Cohn a famous chemist (in1928) prepared a hepatic juice like compound with 100times the potency of normal liver. The primary medicine for pernicious anemia was found in this way. For their principle, in pernicious anemia Whipple, Murphy and Minot, were awarded with Nobel in the year1934 for their great work in the field of physiology.

Discovery of the vitamin B12 came to an end like this. B12 is mainly isolated from bacterial broth. It is a chief water soluble vitamin. In the year 1947, in University of Maryland, Mary Shaw Shorb, were employed for the Poultry Science in a combined assignment with Folkers and Merck. They were given $400 dollar award for the "LLD assay" of $B_{12}$[66].

*Lactobacilli lactis* Darner is called as LLD. A bacterial group needed for the “LLD” preparation which contains the growth promoting substances. Finally the growth promoting substance was named as vitamin. $B_{12}$. Shorb and his assistants utilized the LLD for fast extraction of the anti-pernicious anemia factor from liver extracts. Folkers of the United States and Alexander R. Todd of Great Britain[67], with the support of the chemists Shorb, Karl A,isolated the B12 in the pure form in the

year1948, for which they were respected by the American Society of Nutritional Sciences with Mead Johnson award.

The chemical configuration of VitaminB12 was constructed by Hodgkin and Dorothy **Crowfoot** in1956. It was possible with help of x ray crystallography. The good manufacturing ways were came to market in 1950.[68]

With the sincere work of hundred scientists over eleven years, Woodward and Eschenmoser elucidated the vitamin B12. 1972 they also explained the procedure for isolating the vitamin from liver extract. During the isolation process of vitamin B12, an artefact was found out and it was named as cyanocobalamin. Red methylcobalamin- MeCbl 5'-deoxy-5'-adenosylcobalamin-Adocbl which is yellow coloured are biochemically significant forms of co factors of vitamin B12.

The B12 carrier -proteins studies through X ray crystallography have identified the use of Cbl-based bio conjugates in the imaging techniques. Because of these works Cbl-based bio conjugates are used as vehicles for targeted drug delivery in cancer tissues. It has created a new therapeutic era of vitamin B12.

**Structure**

[70]chemical structures are having a complicated process .The base is corrin ring which has the similarity of HB of porphyrin, cytochrome, chlorophyll. It is centred on a metal cobalt ion. Among the six coordinating places the four are given by the corrin ring. Dimethylbenzimidazole assembly contributes the fifth place. The last place, which is the centre for reaction, which is adjustable, so that hydroxyl (-OH), a cyano (-CN), a methyl groups ($-CH_3$) or a Ado cbl'- here the fifth carbon in the deoxyribose sugar get attaches covalently with Co. consistently, the four $B_{12}$ arrangements are made in this way' the first bond explained in biology is C-CO which was explained during the elucidation of B12 structure only. Enzymes hydrogenases related with cobalt use, includes metal-carbon bonds.

Vitamin $B_{12}$ is a collective description given to represent the group of corrin ring and cobalt. The hydrogenases are required by these elements for their accurate vitamin role in the body. All the compounds having, cobalt-corrin fragments, has to be synthesized by the bacteria.

By way of enzymatic exclusion of certain prosthetic organic groups from the cobalt atom, the human can transform any form of $B_{12}$ to its

active form. Due to the nature of cobalt ring, all the vitamer forms are in deep red colour only.

Cyanocobalamin is **“**vitamer"[71,] form of B12. Actively metabolised to its co enzyme form in the body. On the other hand, the cyanocobalamin is not classically present in nature. The methyl and adenosyl forms creates strong attachment with cyanide (-CN). The cyanocobalamin of $B_{12}$ is crystallized easily. It does not undergo air-oxidation. Cbl is used as food additive in all the commercially available multivitamin preparations. Pure form Cbl are seen in dark pink colour is due to extreme octahedral cobalt(II) compounds and the minerals can be simply powdered to millimetre size.

The Co atom of the Cbl is correctly coordinated with seven amide side chains and by corrin ring (*a*-*g*). The numerical and assembly of the atoms and the complicated molecular arrangements were revealed by Hodgkin Dorothy .

The corrin ring includes four oxidized pyrrole rings, A-D, having a straight link among A and D, rings having helical solitary complete *R*-arrangements in its spiral midpoints between C19- C1. The side chain is connected through an amide bond with nucleotide with the support of benzeimidazole 5-6 which harmonizes Co at the axial spot on the side of corrin. Cobalamines+3are (octahedral) are due to oxidized state of cobalt

base-on form synchronised with the axial X ligand with the left arm of corrin ring. On protonating the NB3 atom with benzimidazole relations Cobalamins can be proceeds the base-off form changed through endogenous ligand.

Oxidized form of co (II) is presented without a axial ligand and commonly recognized as cobalamin II or B12, but in co (I) form it is seen as the tetra coordinated base off form. Coronoids will have the structural modification depending on the cobalaminss,

CNCbl, does not have direct organic functions while MeCbl and AdoCbl(X=5'deoxyadenosylcobalamins) act as prosthetic, group for many enzymatic reactions. The cofactor role is due to the metal bond of corbon enzymatic catalytic replies are depends on the breaking and aking (Cobalamins) of the Co-C bond.

MeCbl is the coenzyme of numerous methyl transferases, such as methionine synthase, meant for the synthesis of methionine in bacteria and humans. Methyl corrinoids, are the coenzyme in the carbon dioxide fixing path ways in the aerogenic methanogenic bacteria

The cofactor every time fixes the Apo enzyme in the base-off form and, in a subgroup of methyl transferases, the Co is co-ordinated through a histidine scaffold in the /His-on binding protein [8] of base.[1]

[75] For eliminases and isomerases,AdoCbl is the cofactor- which moves one -2 a H atom from a electronegative cluster to the proceeding carbon like L-methylmalonyl-CoA-mutase-.MMCM Catalyses revocable conversion of to the succinyl COA L methylmalonyl COA remainder in bacteria and mammals.

The only adenoxyl cobalamine containing enzyme in the body is methylmalonyl COA mutase. In the nucleotides Ribonucleotide reductase class II MMCM is the cofactor. Co-Ado bond cleavage is done only through the adenoxy cofactor sulphidogenic microbes (are used to de chlorinate aliphatic and aromatic chloro-hydrocarbons) uses coblamines as coenzymes.

B12 group of coenzymes consists of Fe4S4 and Fe3S clusters along with corrinoid. The corrinoid, is a novel cofactor for tetra chloral ethylene. The quite interesting story of the 2 cofactors MeCbl and AdoCbl is the Co-C bond, is widely taken into account as its cleavage is interesting role in the pathways of many metabolism.[75]

**B12 Dependent Enzymes**

**Methyl transferase**

[76]Corrinoid methyl transferases designs have been explained. It is explained through 3 models by Mathew's rowina.

[78]The principal form, Co (I) of the corrinoid résumés a methyl group from the substrate methyl-donor (D-CH) n the external module and Co(III)-CH transfers its methyl to the methyl-acceptor (A) in the external module to give the methyl acceptor product (A-CH3)

**MeCbl methionine synthase (MetH)**

From homocysteine methionine is synthesized by shifting of a methyl group fromN5-methiltetrahydrofolate. This reaction is biocatalyzed by methionine synthase to form MeCbl and Hfolate, this shifts the methyl group to Hcy to regenerate cobalamin (I) and to form methionine.

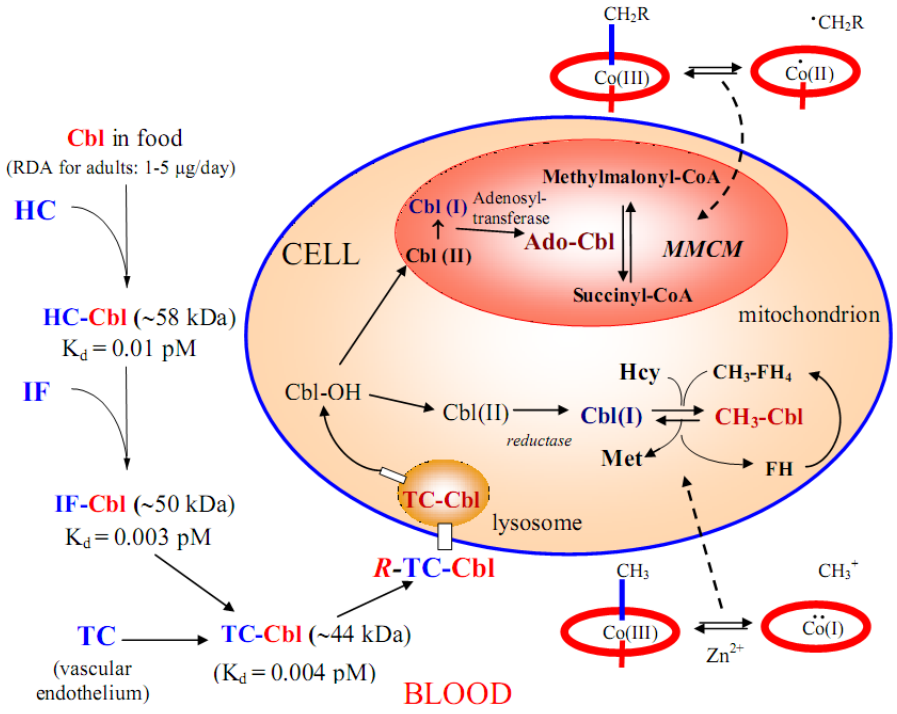



Figure shows creation and character of the cofactors and MeCbl, AdoCbl heterolytic and homolytic sepration of the Co-C bond in MeCbl

Ado Cbl Ado Cbl enzymesare correspondingly demonstrated. The consistent arrow specifies the site of changes, heterolysis into the cytoplasm and homolysis into the mitochondria.

**5. Absorption& Cellular Uptake of B12**

Higher organism does not have the capacity to synthesize cobalamines in the body as they are lacking the necessity enzyme system. They have to be supplemented in the food compulsorily. If the cobalamines are not taken in the body or if there is defective absorption in the enzymatic catalysis may aggravate nervous system disease, in adding tovitaminB12 deficiency anaemia.

In humans the vitamin B12 follows complicated path way for the intestinal uptake, different mode of transport, cellular utilisation. The carrier proteins, haptocorrin -HC, transcobalamin- TC intrinsic factor (IF). They make close-fitting compounds through Cbl.

The dietary cynocobalamin is exclusively fixed to haptocorrin-HC which is a salivary protein and forms HC-Cbl. In the duodenum the proteases of pancreatic origin splits into HC and Cabalamine. Then the vitamin is fixed with castles factor. Intrinsic –B12 factor compound formed. Within the intestinal cells, the intrinsic factor is released. Now the vitamin attaches itself tightly with Transcobalamin-Cbl complex. The

vitamin undergoes endocytosis in the plasma membrane, and the transcobalamin is free for next cycle.

Within the hepatic tissues TC- undergoes exocytosis, freeing B12. By a special process methyl-Cbl (in cytoplasm) 5 -deoxyadenosyl-Cbl( in mitochondria) is formed.

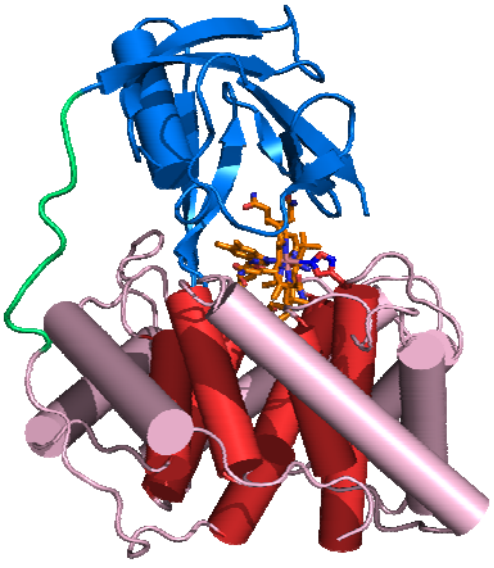
Currently the researchers have found the truth that the surface binding affinity of B12. This property is used in the targeted drug delivery in tumour tissues.

**Transport proteins in mammals**

Three carrier proteins are available for the transportation namely HC IF and TC. All are peptides. But, HC, IF – undergoes glycosylation. All transport protein transports a solitary molecule which forms firm attachment with B12 brings a single Cbl molecule which is firmly bound. Nevertheless, carrier proteins differs in their specificity in the order HC << TC < IF. Up to 2006, the carrier protein crystallography was not conducted though structural study of the enzymes was started in the year 1994 itself.

The configuration of human and bovine TC-Cbl compounds are identical and contains of twoα and β domains joined together with a linker.

"The αβ sphere structure of transcobalamin (TC) and human intrinsic factor (IF) are similar. The picture displays bi-domain assembly of TC. The interior six α coils are in red and the six exterior ones in violet[84]. The β sphere is in blue. The loop linking the spheres is in green. b) The bi -domain construction of IF; the inner six α helices are in red and the six peripheral ones in violet. The β sphere is in blue."



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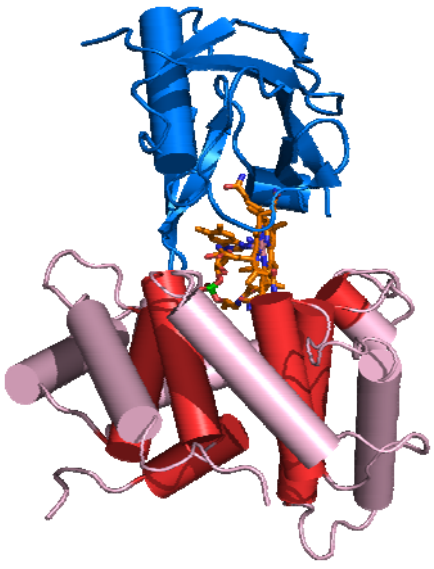


B

For long period, the transport and the absorption vitamin B12 in human has recommended the usage of Cbl to increase the bioavailability of proteins and drugs with reduced solubility and minimal gut absorption.[83]

Currently vitamin B12 is used in the delivery (proteins peptides) of hormones and EPO, more newly, insulin. Furthermore, rapidly proliferating cell likes cancer tissues needs greater quantities of Cbl comparing to normal tissues, added this property in designing the bio conjugates to transfer NMR radio imaging drugs or cytotoxic medicines to the tumour tissues.[84], functioning conjugates can be engaged as "Trojan horses"[85] to transport the diagnostic label or anti-tumoural into the neoplastic site.

Cobalamins are the biochemical name for the B12 vitamer. The interesting fact about cobalamins, are -their photosensitivity. They are soluble in water. B12 is essential for the growth, maturation of the RBCS and also the neurons. . cobalamines are the coenzymes in the transformation of homocysteine to methionine. It is again the co factor in the synthesis of suuccinyl COA from methyl coA

**B12 Sources**

Humans and higher animals have to synthesis directly or indirectly from bacteria.

These bacteria are habituated in terminal gut where $B_{12}$is absorbed

Animal protein

Fish and shellfish liver, meat

Eggs, poultry products milk,

*Chlorella*, fresh-water single cell green algae,. to contain biologically active$B_{12}$

Fermented black tea which is familiar in Japan called as Batabata-cha is having the biological potent form of $B_{12}$

**Risk conditions**

Strict vegetarians, those who are on certain prescriptions, patients with assured gastro- intestinal disorders, the persons having poor food intake habit, and aged persons in the range of 60-80.

Poor nutritional intake defect in the stomach, intestinal and terminal ileum can cause deficiency of the B12.

With references to the causes, the prevalence of deficiency ranges from 5–60%. In general it is 20% only. The vitamin deficiency is not specific. It is vague so identifying the symptoms in the early stages is very difficult as this is the only water soluble vitamin stored in the liver. The deficiency manifestations will take at least 3-4 years to appear.

**Common Symptoms of B12 Deficiency**

Paraesthesia, weakness, numbness,

- Neuropathic
- Ataxia, Abnormal gait
- Myelopathy
    - cerebral causes ;
    - Depression
    - Memory loss
    - Abnormal gait

Anaemia (Haematological -not so common)

Deficiency for a prolonged period ends in the neuronal complications like death of the neurons, demyelination, axonal changes and apoptosis. So the interventions should be done promptly.

**RDA FOR VITAMINB12 IN ADULTS**

| Age | Sex/male µgms/l | femaleµgms/l | Pregnancy µgms/l | Lactation µgms/l |
|---|---|---|---|---|
| 19&older | 2.4 | 2.39 | 2.6 | 2.4 |
| 50& older | 2.4 | 2.39 | - | - |

## SCREENING

There are no official recommendations for the routine screening of B12. Guidelines direct the screening in risk persons. Patients are according to the risk advised for screening and management. Patients with history of malnutrition auto immune disorders genetic defects in the absorption are advised to undergo screening requlary.[89]

One suggestion is the people above the age of 50 are advised to undergo annual screening every five years up to sixty five years. After that annually once up to the age of 80. To determine the status of the vitamin in the body , the serum levels itself is taken as a marker recently. The highly sensitive marker for the vitamin levels in the body is holotranscobalamin, Halo TC. But luckily the commercial use is almost not available. .

The reference range is still a debate. In India it is usually 240 µ/l-900µ/l were normal[22]. This is due to most of them are vegetarians and the lower socio economic status. Below 240 pmol[23] is considered as deficiency status. The serum MMA, homocysteine, folate levels can affect the body status of the vitamin. Diseases like coeliac atrophic gastritis, liver problems of chronic durations, bone disorders and malignancy will also alter the level. MCV used as a marker of the

deficiency status is not acceptable as it is not as sensitive, as it misses 90% of the deficiency status.

**DRUGS**

- Alcohol: Extreme alcohol consumption more than 15 days can reduce vitamin $B_{12}$ absorption.
- Amino salicylic acid - PAS, para-aminosalicylic augments the absorption of oral preparations level up to 55%. During the course of treatment Megaloblastes are noticed. Unusual anaemia's also presented in the peripheral blood films. If the dose is more than 12gms or the duration is more than one month whichever is early then the individual must be screened for the vitamin levels.
- Antibiotics: drugs such as metronidazole will increases the B12 level. This response of the drugs over the microbial in the gut is controversial yet it has to be proved.
- Oral contraceptives on $B_{12}$ serum status are contrasting each other. When oral contraception is stopped, the vitamin $B_{12}$ levels returns to usual levels.
- Cobalt radiation

- During any form of radio therapy gut loses the capacity to absorb the vitamin.
- Colchicine:> 3.9 mg/ over 24 hrs causes mucosal atrophy and there by a chances of defective absorption. This will take a minimum period of 3 years.
- So screening is advised if colchcine is continued for more than three years in mega dose.

**$H_2$-receptorantagoanists**

$H_2$ blockers will interfere with the dietary forms. Supplemental forms are not affected by these drugs. Gastric juice is needed to free, dietary salivary protein bound forms. As acid levels are reduced by these drugs monitoring of the B12 levels are suggested if the therapy is continued for unusual durations.

Metformin-Glucophage – definitely reduces the capacity of the cubilin receptors which is dependent on calcium uptake. Sustained use will leads to B12deficiency and. hyperhomocysteinemia, by interfering with folic acid metabolism through the co enzyme the raised homocysteine which is "individual risk[90] for cardiovascular disease, particularly amongst those with type 2 diabetes."

- Infrequent form of megaloblasts has demonstrated in the individuals those who are taking the biguanides for 6 years are more. The incidence of drug induced vitamin deficiency is about 22-30% in these persons.

- If the nutritional status of the individual is satisfactory the deficiency is doubtful. The supplementation of calcium also revert the drug induced deficiency status. If adequate formulations are taken then the deficiency is not occurs even in chronic course of the doses.

- Neomycin: though it interferes with the cellular uptake of the vitamin at the intestinal levels. Pernicious anaemia occurs if mega therapy is continued for longer periods. Supplementation is not needed in the regular doses.

- Nicotine: -decreases $B_{12}$ levels. The requirement of oral formulas is yet to be proved.

- Nitrous oxide: because of oxidation the Cobolamine are become incapable for enzymatic reactions myelopathy, sensory neuropathy, encephalopathy occurs according to the vitamin status of the individual. The response to treatment therapy is very slow though high doses are supplemented.[91]

**Phenytoin, Phenobarbital, Primidone:** These anticonvulsants are related with defective absorption of B12. In CSF and the serum the levels are decreased. So the megaloblastic anaemia takes the upper hand.

**Certain drugs can also disturb B12levels**.

The sustained use of metformin for diabetes can drop B12levels adequately to gain the clinical significance. Sometimes the neuropathy of T2DM can be considered as neuropathies of the vitamin deficiency. Both are separate issues.

# MATERIALS AND METHODS

This research was conducted during the period April 2015– August 2015 as a cross sectional comparative type in the department of Diabetology, department of Biochemistry in Kilpauk Medical College Chennai.

## STUDY POPULATION:

- **CASES:**

45 patients of known type II diabetes mellitus on metformin for a period of 12 months will be selected as cases from OPD of Department of Diabetology, Govt. Kilpauk Medical College & Hospital (GKMCH), and Chennai.

**CONTROLS**

- Control group consists of 45 known persons with T2DM not on biguanides like metformin in the past one year. Controls are age and sex matched.

- **INCLUSION CRIETERIA**
- 40 to 60 years of Previously diagnosed T 2 DM
- Both genders (female & male) are included.
- Those who are on insulin & other OHA drugs also included.

- **EXCLUSION CRITERIA**

  - History of anaemia,
  - Prior transfusion,
  - Renal insufficiency,
  - Thyroid illness,
  - Alcohol intake,
  - Prior gastric surgery, proton pump inhibitors, those with malabsorption syndrome

The study was approved by the Institutional Ethical Committee of GKMC, Chennai. After a full explanation of the study a written informed consent was obtained from each participant.

**SAMPLE COLLECTION:**

- 3 ml random venous blood sample was drawn from ante cubital vein of patients, after fulfilling selection criteria in a plain test tube.

Sample is separated by centrifugation at 3000 rpm for 15 minutes; within 2hrs of collection separated serum is stored at -20 °c for further analysis.

## Estimation of Plasma Glucose:

**Method**: Glucose Oxidase peroxidase (GOD / POD )( End point Test )

**Kit used**: Erba

**Principle:**

$$\text{Glucose} + O2 + {}_{H2O} \xrightarrow[\text{Peroxidase}]{\text{Glucose Oxidase}} \text{Gluconic acid} + H2\,O$$

$$H2O2 + 4APP + PHENOL \xrightarrow{\text{Pink coloured complex}} \text{QUINONEIMINE COMPLEX} + H2O$$

a. Pink coloured Quinonemine complex is developed to depending on the glucose concentration in the sample. Absorbance was read at 505 nm.

**Composition of reagents**

**Reagent -1: Enzyme reagent**

Peroxidase ->2000U/L

Glucose oxidase - 20000U/L

Phosphate buffer - 200 mmol/

L Phenol - 10 mmol/L

**Glucose standard** - 100 mg/dl

**Procedure**:

10µl of plasma was added to 1000µl of working reagent and incubated for at 37.c° 15 minutes.

## Reference range:

Fasting plasma glucose -70- 110mg/dl

## Blood Urea Estimation

**Kit used**: Accucare

**Method**: Urease - GLDH (kinetic UV test)

**Principle:**

Urea is hydrolysed to Urease in the presence of water which produces ammonia and carbon dioxide. In the presence of glutamate dehydrogenase the ammonia joins with NADH and oxoglutarate to give glutamate and NAD.

$$\text{Urea} + H_2O \xrightarrow{\textit{Urease}} 2NH_4^+ + H_2O$$

$$NH_4^+ + \text{NADH} + H^+ + \text{2-oxoglutarate} \xrightarrow{\text{GLDH}} \text{Glutamate} + \text{NAD}$$

The rate of reduction in absorbance is initially directly related to the amount of urea in the solution. The readings are taken at 340 nm.

**Composition of Reagent**

Reagents I: Buffer reagent

Reagent II: Enzyme reagent

Urea standard: 50 mg/dl

**Preparation of reagent**

1ml of Enzyme is taken along with4 ml of Buffer reagent both are mixed gently.

**Methods**

10µl of plasma is added to 1000µl of reconstituted reagent and absorbance measured at 340 nm. After 30 secs and 60 secs the rate of reduction is directly related to amount of Urea in the solution.

**Reference range**: Serum / Plasma Urea → 15 – 39 mg/dl.

**Creatinine Estimation:**

**Kit used**: Erba

**Method:** Jaffe's method (Picrate Method) (Initial rate method)

## Principle

Creatinine when mixed with alkaline picrate after a chemical reaction it produces an orangish – yellow colour which is called - Jaffes reaction Colour of the absorbance is directly related to the amount of creatinine in the given solution. Absorbance is measured at 500nm.

**composition**

**Reagent 1**: picric acid reagent

Picric acid - 25.8 mmol

**Reagent 2**:

Sodium hydroxide – 95 mmol/L

Sodium hydroxide reagent

**Standard**

2mg/dl (0.166 mmol/L)

**Preparation of reagent**

Both the reagents are mixed with equal volumes. After 15 minutes, it is used.

**Procedure:**

100µl of the sample is added to 1000µl of the reconstituted reagent and mixed gently and immediately the reading is taken. Difference in the

initial absorbance after 20 secs and final absorbance after 80 secs of correct mixing, the readings are taken.

## Reference range:

Male: 0.6 – 1.1 mg/dl,

Female: 0.5 – 0.9 mg/dl.

## Human Vitamin B12- ELISA

### Uses of the kit

The kit is based on sandwich technique of ELISA for the measurement of HumanVB12 in biological fluids

## COMPONENTS

### Reagents Quantity

| | |
|---|---|
| Pre-coated, ready to use 96-well plate | 1 |
| Calibrator Diluent | 1 × 20 mL |
| Calibrator (lyophilized) | 2 |
| Detection Reagent A | 1 × 120 µL |
| Detection Reagent B | 1 × 120 µL |
| Assay Diluent A | ( 2x concentrate) 1 x6mL |
| Assay Diluent B | (2X concentrate) 1 × 6 mL |
| Stop Solution | 1 × 6 mL |
| TMB Substrate | 1 × 9 mL |

| | |
|---|---|
| Wash Buffer (30X concentrate) | 1 × 20 mL |

Plate cover for all the wells

**REQUIRED MATERIALS**

- Eppendorf Tubes for diluting samples.
- Deionized or distilled water
- Precision single and multi-channel pipettes and disposable tips.
- Container for Wash Solution
- Absorbent paper for blotting the microtiter plate
- Micro plate reader with 450 ± 10 nm filter.
- **STORAGE**

**PRINCIPLE**

According to their label all reagents are stored. The **Calibrators Detection Reagent B Detection Reagent A** and the **96-well plate are** stored in the deep freezer at -20°C. The additional strips are kept in a protected bag to avoid the atmospheric air. Opened kits are stable, till the expired period if kept correctly.

The microtiter wells are pre- treated with VB12 specific antibody samples and to the exact microtiter, the exact calibrators a with a specific VitB12 biotin-conjugate are added.

Horseradish Peroxidase (HRP) avidin is then added to each micro well after the correct incubation period. Then substrate of TMB is added. Now the vitaminB12 conjugate undergoes reaction with avidin conjugate which will show a colour change. The substrate enzyme reaction is resolved by addition of sulphuric acid. The colour changes are read spectrophotometric ally at a 450 nm ± 2 nm wavelength. The VitaminB12 levels in the test solution are determined by comparing the O.D. of the calibration curve to the test solution curve.

## STORAGE OF COLLECTED SAMPLE

### Serum

The samples which were collected in the plain test kept for 2 hours in the room temperature for clot formation. Then centrifugation done at1000 x g for 20 minutes .Serum separated freshly is used immediately or transferred in aliquots and kept in deep freezer in -20°C or -80°C to future use. Repeated freezing are avoided

**Note:**

1. The samples stored in2-8°C,are used within 5 days, otherwise they are kept at deep freezer 20°C -80°Cto elude contamination and to maintain the potency.

2. Samples are brought to room temperature then assay is done.
3. As haemolysis will impact the result. (Haemolysis must be avoided)

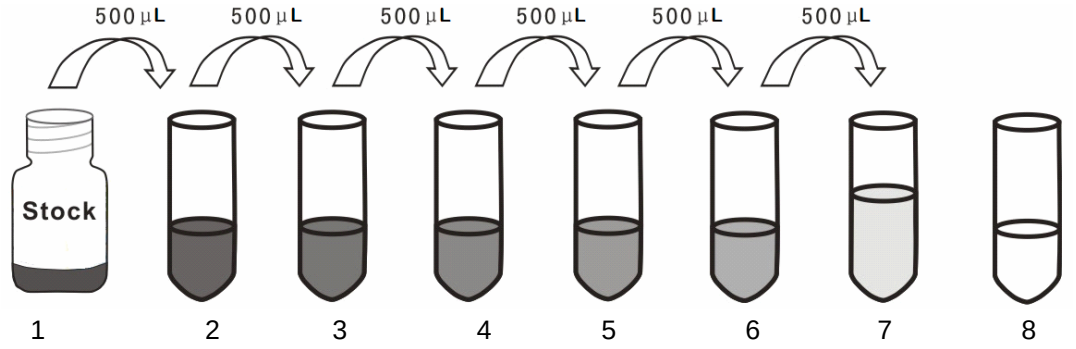
**PREPARATION OF REAGENT**

Samples and all components in the kit are allowed as such for (18-25°C) 20 minutes.

**Calibrators**

The Calibrator are mixed with respectable diluents as per the guide lines and allowed for the incubation time of ten minutes. By mixing slowly and gently foaming can be avoided. The stock calibrator is now at the concentration 1000 pmol/L. With the help of Diluent and the stock solution of the calibrator's different range of the calibrators are prepared. From the stock Calibrator 500µl is added to six tubes and different concentration of sub stock of prepared (as given below fresh tip is used

for each delivery and every tube is mixed completely before subsequent delivery. calibrators will have seven concentrations of 1,000, 500, 250, 125, 62.5, 31.2, 15.6, pmol/L, final tube contains only the Diluent will be the blank at 0 pmol/L..




**Tubes**

**-pmol/l**1000 500 250 125 62.5 31.5 15.6 0

Assay Diluent A and B Six mL of deionized water six ml of Assay Diluent A is added so twelve mL of working solution is made. Then with same procedure with help of Assay Diluent WORKING SOLUTION OF B is prepared. Both are kept correctly as per the guide lines.

**Detection Reagent B AND A**

Both detection reagents A &B are spinned. By diluting the working **Assay Diluent A or B, sub concentrations are prepared** individually (1:100).

**SOLUTION FOR WASH**

By adding twenty ml of wash solution to five eighty ml of distilled water the solution for wash is prepared.

**TMB Substrate**

Only the required substrate is pipetted with fresh tips and the remaining solution are discarded, not returned to bottle meant for substrate

**Notification**

Reagents are not dissolved at37°C. 1

1. The calibrators are. Prepared within 15 minutes before the assay commencement

2. The dilutions are added not directly to the micro plate.

3. Calibrators and the working Reagent A, B are added as per the instruction. As per the recommendations the reconstitution is done. Care is taken to see whether the crystals are dissolved fully or not. Foaming is avoided during the procedure. To reduce pipetting inaccuracy small volumes are used. Pipettes with calibration are used for the same purpose. Every time only ten µl is pipetted.

4. Diluted Calibrators, all the Reagents are for single time use.

5. The Wash Solution (30X) must be free of crystals. If not the solution is slowly mixed till it is dissolved fully.

**PROCEDURE**

Before starting the assay the concentration of vitamin b12 is estimated. If the values are not seen in the detection limit of calibration curve, dilutions are done. Correct wells are kept for proper diluted calibrators, blank and test. For calibrators, seven micro wells are prepared

One well for blank is kept separately. Blanks and calibrators are added in to the concerned micro wells then the test is added to the remaining microtiter plates. Hundred µL of e Detection Reagent A solution is added to every micro plate after covering with silver paper incubated at 37°C for 2 hours.

2. From each well, the liquid removed not washed.

3. The solution is aspirated first then and each well is washed with four hundred µL of Solution for wash ,with a wash bottle, wash can done manually or automated washer with for one –two minutes till all the micro plates are dried fully. Invert all wells on a filter paper. 3 times this is repeated for. After the last wash any drop of liquid missed is removed by Wash Buffer aspirating or decanting.

4. The plates are inverted against a filter paper.

5. To working solution, 100 µL of Detection Reagent B is added to each well. After covering with the silver paper incubated for thirty minutes at 37°C

6. The wash procedure and aspiration is done repetitively for five times like the previous procedure.

7. **Substrate ninety µl is added** to every well after covered proper coverings at 37°C incubation done for 15 - 25 minutes (The solution will turn blue after the addition of Substrate Solution. Protect the wells from light.

8. To each well 50 µL of **Stop Solution** is added. Now the liquid will turn yellow. The side of the plate is tapped slowly so the liquid is mixed gently. If the colour change is not uniform, the plate is gently tapped to verify full mixing.

9. Fingerprints drops of solution or on the bottom of the plate are removed and confirmed, the surface of the liquid is seen without any bubbles. Then the plates are kept in the micro plate reader and measurements are taken at450 nm reading is taken at once.

## RESULTS

The duplicate readings for each calibrator, control, and sample are averaged and subtracted from the average zero calibrator optical density. A calibration curve a calibration curve is produced by manoeuvring of mean absorbance y-axis against the concentration. A straight line made between the points. The statistics may be corrected by plotting the log of the VB12 concentrations versus the log of the O.D

## PERFORMANCE

### Detection Range

The detection range is: 15.6 – 1,000 pmol/L.

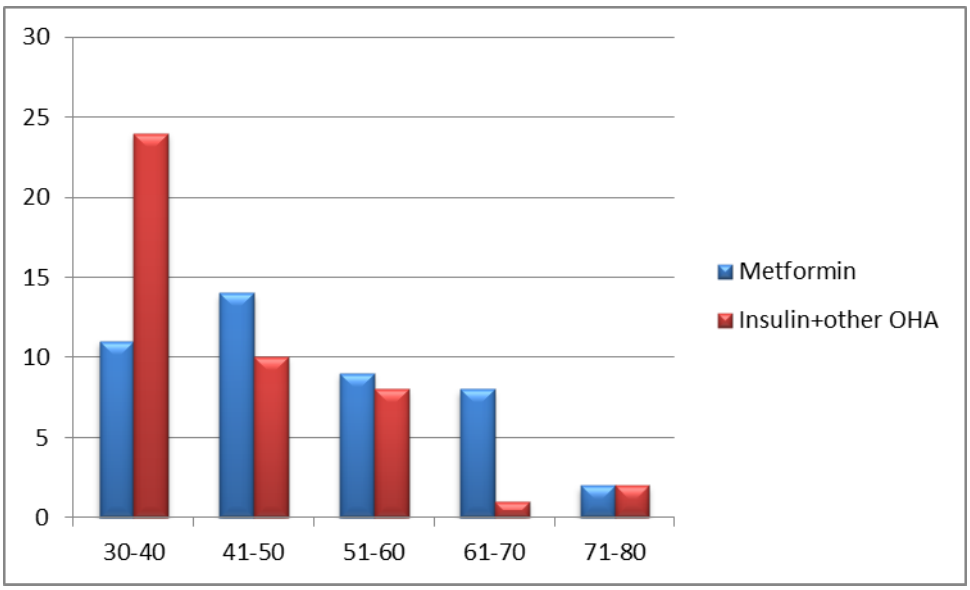
The calibration curve concentrations used for the ELISA's were 1,000, 500, 250, 125, 62.5, 31.2, 15.6 pmol/L.

### Sensitivity

The lowest measurable value of Human VB12 is classically less than 3.9 pmol/L. Lower Limit of Detection (LLD) or the sensitivity of this assay, is demarcated as the lowest amount of concentration that could be renowned from zero. It was determined by the mean O.D. value of 20 duplicates of the zero calibrator plus three standard deviations.

# RESULTS AND STATISCTICS

1. Age wise distribution of participants among study groups




| | Group | N | Mean | Std. Deviation | Std. Error Mean | P value |
|---|---|---|---|---|---|---|
| Age in years | Control | 45 | 43.31 | 10.377 | 1.547 | .006 |
| | Cases | 45 | 49.96 | 12.019 | 1.792 | |

Age based distribution into Test and Control groups were also statistically significant. Insulin was used predominantly among patients between 30 to 60 years in our study, but metformin use among test group participants was widely dispersed.

2. Duration of Diabetes among study participants

| | Group | N | Mean | Std. Deviation | Std. Error Mean | P value |
|---|---|---|---|---|---|---|
| Duration of DM | Control | 45 | 5.1333 | 4.27253 | .63691 | .632 |
| | Cases | 45 | 5.5111 | 3.08679 | .46015 | |

No statistical significance was obtained with regards to duration of diabetes pharmacotherapy between control and test group

**3.** Independent t-test between control and test with regards to glucose levels

| | Group | N | Mean | Std. Deviation | P value |
|---|---|---|---|---|---|
| Glucose | Control | 45 | 138.242 | 25.9523 | 0.575 |
| | Cases | 45 | 142.331 | 41.2443 | |

In our study conducted among 45 Test (Receiving Metformin) and 45 control (Receiving Insulin other anti-diabetic medication), no statistically significant inter-group variation existed with regards to glycaemic levels during recruitment.

4. Variation in RFT between control and test (independent t test)

Urea

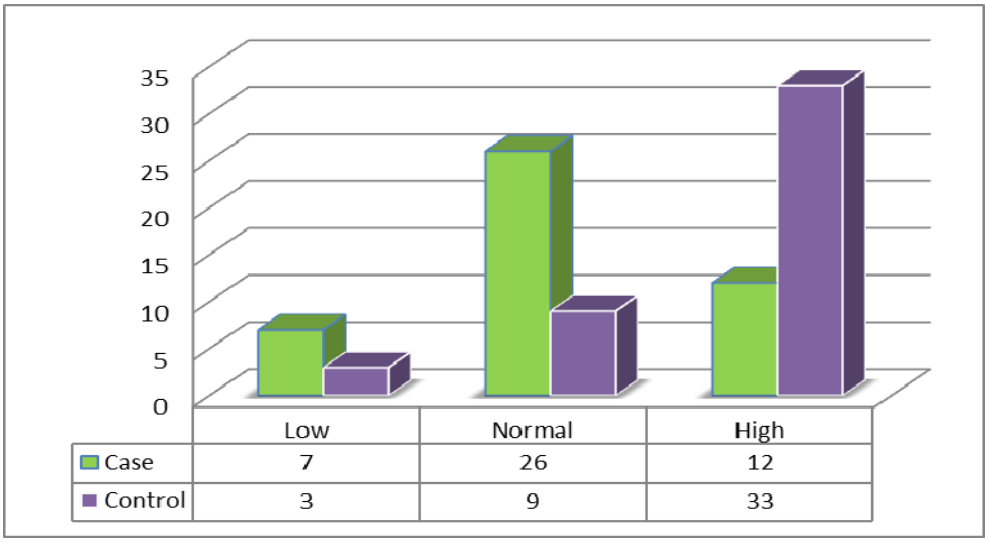
| | Group | N | Mean | Std. Deviation | Std. Error Mean | P value |
|---|---|---|---|---|---|---|
| Urea | Control | 45 | 27.160 | 6.3434 | .9456 | |
| | Cases | 45 | 28.178 | 8.7029 | 1.2974 | .528 |

Creatinine

| | Group | N | Mean | Std. Deviation | Std. Error Mean | P value |
|---|---|---|---|---|---|---|
| Creatinine | Control | 45 | 1.0211 | .26128 | .03895 | |
| | Cases | 45 | .8663 | .40739 | .06073 | .035 |

Between the test and control group, significant intergroup variation was seen in creatinine value, but not in urea levels when independent student's t test was applied separately for each parameter.

## 5. Difference in B12 levels between cases and controls



In this among the controls 3 had the low vitamin level and 9 had normal level 33 had high level which comes in the following % respectively7.6,21.4 &69.8where as in cases it is16.5 42.6, 34.6so metformin group had noticeable vitaminB12deficiency prevalence comparing their control.

| | Group | N | Mean | Std. Deviation | Std. Error Mean | P value |
|---|---|---|---|---|---|---|
| B12 | Control | 45 | 1111.202 | 414.7199 | 61.8228 | <.001** |
| | Cases | 45 | 754.504 | 395.7267 | 58.9915 | |

When study participants were analyzed for intergroup differences in B12 levels, the mean difference in B12 levels between Control (Insulin + non biguanide OHA) and Test (Metformin) group was 356.70mcg/dl, and when independent t test was applied, highly statistical significant values with p values <.001 was obtained.

6. Correlation of variables in control arm.

| | | Glucose | Urea | Creatinine | B12 | Age in years | Duration of DM | Insulin Dose | Insulin Duration | Other Drug |
|---|---|---|---|---|---|---|---|---|---|---|
| **B12** | Pearson Correlation | .067 | -.010 | .184 | 1 | -.281 | -.449(**) | -.097 | .166 | .310 |
| | Sig. (2-tailed) | .660 | .945 | .225 | . | .061 | .002 | .585 | .357 | .303 |
| | N | 45 | 45 | 45 | 45 | 45 | 45 | 34 | 33 | 13 |

In Pearson Correlation analysis, significant linkage was seen between duration of Diabetes and B12 levels among the 45 patients taking Insulin therapy (with/without other OHA), the other variables were not significantly correlated.

**7.** Correlation of variables in Case arm.

| | | **Glucose** | **Urea** | **Creatinine** | **B12** | **Age in years** | **Duration of DM** | **Metformin** |
|---|---|---|---|---|---|---|---|---|
| **B12** | Pearson Correlation | -.176 | .167 | .021 | 1 | -.287 | -.339(*) | -.572(**) |
| | Sig. (2-tailed) | .248 | .273 | .890 | . | .056 | .023 | .000 |
| | N | 45 | 45 | 45 | 45 | 45 | 45 | 45 |

Among cases (patients taking Metformin), statistically high significant correlation (p value <.001) was obtained between:

- Metformin use and B12 levels (i.e., B12 level decreases as metformin use increases –Negative correlation)
- Similar to control group, duration of diabetes and B12 levels were also positively and significantly correlated.

# DISSCUSSION

In this cross sectional study, 90type 2DM patients were enrolled and they were divided in two groups those who are taking metformin considered as cases where as patients on insulin and other oral hypoglycaemic agents are taken as controls.

The cases on metformin had the prevalence of vitaminB12 deficiency is 16.6% which is significance on regards to controls. It is also dependant on the dose and the duration of drug and the duration of disease. But in the control group the deficiency prevalence is7.6% but in Rah eels Pakistan study conducted in 2013 had a prevalence of 31%which had same results with Marar o and friends of Irish study. The sample size in both populations was large comparing with this study population. Rah eel and his colleague done this study over a period of one year they also studied the methyl malaonic acid level for the sensitivity of vitamin B12.

Our study has limitation since we have not assed the homocysteine level, methyl melanoic acid and the folate levels which will also alter the vitamin status.

Age based distribution in the Test and Control groups were also statistically significant. Insulin was used predominantly among patients between 30 to 60 years in our study, the mean age of the control group is

45 which are 10 years less than Irish and Pakistan study but metformin use among test group participants was widely dispersed.

Between the test and control group, significant intergroup variation was seen in creatinine value. Which are not included in the previous studies. It shows if the glomerular filtration is good, there is a minimal risk of developing B12 deficiency.

In this among the controls 3 had the low vitamin level and 9 had normal level 33 had high level which comes in the following % respectively7.6,21.4 and 69.8where as in cases it is16.5 42.6, 34.6so metformin group had noticeable vitaminB12 deficiency prevalence comparing their control

Irish defined levels of less than 100 pg/ml as vitamin B12 deficiency but as per the Indian guide lines we included 240-900µmol/l[5] among cases (patients taking Metformin), statistically high significant correlation (p value <.001) was obtained between:

- Metformin use and B12 levels (i.e., B12 level decreases as metformin use increases –Negative correlation)

Similar to control group, duration of diabetes and B12 levels were also positively and significantly correlated. Ting et al had done a study in the year 2010and observed the same report. Pflipson et al had the same

result of 22% prevalence, they did the study only through old medical reports.

Ting et al, noticed dose of metformin duration are main factors for Vitamin B12 deficiency .This is similar to our study showing noteworthy association and opposite relation dose, duration of metformin with B12 levels.

Marar O had done the homocysteine and serum methyl malonic acid status and urinary excretion of methyl malonic acid amount also, which are the highly sensitive markers of vitamin B12,they have assessed the neuropathic status also. But they didn't get the consistent findings

**CONCLUSION**

This cross-sectional comparative study concludes that the persons with type 2 DM on chronic metformin therapy showed lower levels of serum vitamin B12 status compared to persons not treated with metformin. This represented that metformin takes a possible risk for vitamin B12 deficiency. The study highlights the need of testing B12 level, when patients are prescribed metformin for long period thereby preventing from its impending side effects.

The study recommends base line B12 levels in high risk patients before starting metformin therapy. It also recommend vitamins screening if therapy is advised for prolonged periods to avoid the neuropathic

complication which are more common in type 2diabetics due to the advanced glycation end products. Doctors treating type 2DM should consider this significant point when treating diabetic patients with metformin, and particularly with those who present with neuropathic signs and symptoms.

## FUTURE SCOPE

Holo transcobalamin is a most specific indicator of cobalamin level for determining B12 vitamin present within the cells, but this is not possible in routine screening. MMA, homocysteine reflects intracellular B12 deficiency, but these compounds may also be elevated in numerous conditions, particularly in reduced renal function. Exclusion criteria were well-defined in this study with regards to increased methyl melanoic acid levels. More studies are needed to support our findings in future.

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# PROFORMA

Name : Age: M/F

OP/IP no : Address :

Occupation :

**Presenting Complaints:**

**Past H/O**:

DM-duration / HT/ Hypercholestrolemia/ Hypothyroid/ IHD/ Endocrine Disorder/ Renal Disease/Hepatic Disease.

**Treatment H/O:** [on insulin, GLP1 analogs,other OHAS, proton pump inhibitors / vitamin supplementation / calcium supplementation/ blood transfusion]

DRUGS OHAS- single/ combination /duration /

**Personal H/O:** smoking /alcohol/tobacco chewing

Family H/o:

**O/E:**

**Built -** obese/thin/moderate **BMI:**

**Height- Weight- Waist-**

Pedal edema /Anemia / Lymphadenopathy-

**Vitals:**

BP: Pulse Rate:

**Systemic examination:**

CVS: RS:

CNS: Abdomen:

**Diagnosis:**

**Investigations:**

Blood sugar –

Sr.Urea :

Sr. Creatinine:

Sr. Vitamin B12

**INSTITUTIONAL ETHICAL COMMITTEE**
**GOVT.KILPAUK MEDICAL COLLEGE,**
**CHENNAI-10**
**Protocol ID No. 09/02/2015 Dt. 26.03.2015**
**CERTIFICATE OF APPROVAL**

The Institutional Ethical Committee of Govt. Kilpauk Medical College, Chennai reviewed and discussed the application for approval "The Prevalance of Vitamin B12 degicieny in Type II DM Patients on Metformin" -submitted by Dr.D.Revathi, III and Year MD., Biochemistry, PostGraduate Student, KMC, Chennai-10.

The Proposal is APPROVED.

The Institutional Ethical Committee expects to be informed about the progress of the study any Adverse Drug Reaction Occurring in the Course of the study any change in the protocol and patient information /informed consent and asks to be provided a copy of the final report.

CHAIRMAN,
Ethical Committee
Govt.Kilpauk Medical College,Chennai

GOVT. KILPAUK MEDICAL COLLEGE
25 SEP 15
Kilpauk, Chennai-10.

25/09/2015

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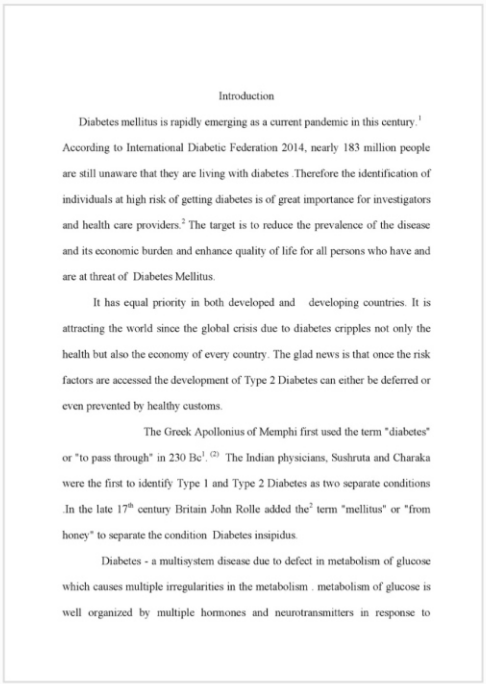

Introduction

Diabetes mellitus is rapidly emerging as a current pandemic in this century.[1] According to International Diabetic Federation 2014, nearly 183 million people are still unaware that they are living with diabetes .Therefore the identification of individuals at high risk of getting diabetes is of great importance for investigators and health care providers.[2] The target is to reduce the prevalence of the disease and its economic burden and enhance quality of life for all persons who have and are at threat of Diabetes Mellitus.

It has equal priority in both developed and developing countries. It is attracting the world since the global crisis due to diabetes cripples not only the health but also the economy of every country. The glad news is that once the risk factors are accessed the development of Type 2 Diabetes can either be deferred or even prevented by healthy customs.

The Greek Apollonius of Memphi first used the term "diabetes" or "to pass through" in 230 Bc[1]. [(2)] The Indian physicians, Sushruta and Charaka were the first to identify Type 1 and Type 2 Diabetes as two separate conditions .In the late 17th century Britain John Rolle added the[2] term "mellitus" or "from honey" to separate the condition Diabetes insipidus.

Diabetes - a multisystem disease due to defect in metabolism of glucose which causes multiple irregularities in the metabolism . metabolism of glucose is well organized by multiple hormones and neurotransmitters in response to

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